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14. ABSTRACT

Our studies to date have determined that human oogonial stem cells, while far less stable than their murine counterparts, can be successfully expanded and propagated in culture. This has allowed us to begin testing the potential tumorigenicity of these cells with the ultimate goal of comparing the DNA signature of the oogonial stem cell-derived tumors to that of primary human ovarian cancer. We have also successfully introduced in human oogonial stem cells genetic alterations commonly detected in ovarian cancer. We are now generating tumors from these altered oogonial stem cells and will compare the histologic features in the tumors formed from the modified oogonial stem cells to those of primary tumors collected from women diagnosed with serous ovarian cancer. Also of importance is the identification of Ddx4-positive cells in xenografts derived from primary human ovarian serous tumors. The biological significance of this apparent rare population is yet to be determined. We have designed and implemented initial studies to test the relative tumorigenicity of Ddx4-positive, CD133-positive and Ddx4 CD133 double positive fractions. Demonstrating that these highly specialized human oogonial stem cells have the capacity to form ovarian tumors would be a major paradigm shift.

15. SUBJECT TERMS

Serous Ovarian Cancer, Cell of Origin, Human Oogonial Stem Cells, Tumorigenicity, Ddx4

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INTRODUCTION:

High grade ovarian cancer remains one of the most deadly gynecologic cancers. The high mortality rate is often attributed to our inability to detect early stage disease, the high incidence of recurrent chemoresistant disease and our limited understanding of the biology underlying early events in ovarian cancer development as reflected in continued debate surrounding the cell and site of origin of ovarian cancer. Our preliminary data generated using oogonial stem cells derived from the mouse ovary suggested that these rare cells have the capacity to form tumors in immunocompromised mice when 'misplaced' outside of the ovary. Specifically, while no tumor developed when oogonial stem cells were injected directly into the ovary, injection of the same cells into the mouse abdominal cavity resulted in tumors that histologically resemble ovarian cancer. Subsequent studies have identified the presence of a similarly rare oogonial stem cell population in the human ovary. We hypothesize that human oogonial stem cells either shed from the ovarian surface during ovulation or naturally present in an aged ovary have tumorigenic potential. Furthermore, we believe a genetic misprint will allow these cells to undergo additional changes common to the different ovarian cancer subtypes. Our objectives, therefore, are to determine the tumorigenic capacity of human oogonial stem cells and compare the DNA signature of the oogonial stem cell-derived tumors to that of primary human ovarian cancer. In addition, we will induce in human oogonial stem cells genetic alterations commonly detected in ovarian cancer and compare the histologic features in the tumors formed from the modified oogonial stem cells to those of primary tumors collected from women diagnosed with serous ovarian cancer. Demonstrating that these highly specialized human oogonial stem cells have the capacity to form ovarian tumors would be a major paradigm shift.

BODY:

Task 1: Tumorigenic assessment of OSCs isolated reproductively aged and menopausal women.

Subtask 1a: In Aim I human OSCs isolated from a total of 10 patients (5 reproductive age women and 5 peri-/post-menopausal women) will be expanded in vitro under conditions established in our laboratory.

Unfortunately, both the ovarian samples and oogonial stem cell preparations/cultures isolated from pre- and post-menopausal ovaries obtained prior to receiving this funding did not survive long-term cryopreservation. We therefore had to modify our cryopreservation methods to ensure viability of both the ovarian samples and more importantly, the isolated oogonial stem cell cultures. Following optimization of this process, we have actively worked with MGH GYN Tissue Repository staff to accrue new samples and to restock the ovarian tissue required for the proposed studies. Since receiving the award, we have acquired, via the MGH GYN Tissue Repository, human ovarian tissue from nine different patients who fit the relevant clinical criteria. A subset of these ovarian samples has been processed to generate oogonial stem cell cultures. The remainder has been cryopreserved for future isolation of oogonial stem cells. The collected samples comprise ovarian tissue from pre- and post-menopausal women. As expected, the majority of these samples (8 of 9 samples) are from post-menopausal women. We will

continue to accrue samples in order to obtain sufficient numbers of ovaries from pre-menopausal women.

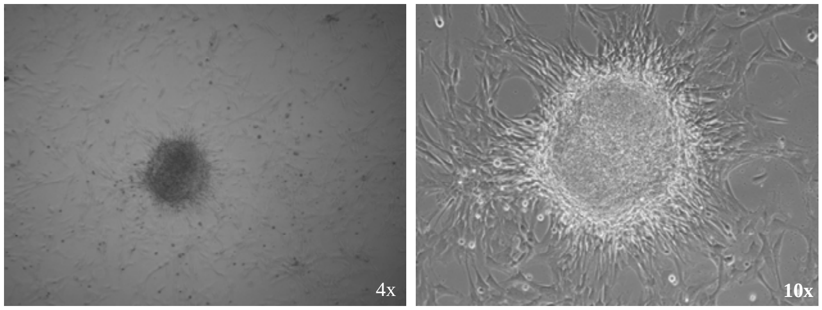

Figure 1. OSC colony formation following ex vivo expansion. Ddx4+ OSCs isolated from an ovarian sample obtained from a 57 year old post-menopausal patient were expanded in culture. Shown here are images of a colony that formed 17 days after sorting. Left panel, 4x; right panel, 10x.

Three of the newly acquired ovarian samples were subjected to cell sorting to generate Ddx4-positive oogonial stem cells. Cells obtained from two of the three sorted ovarian samples were healthy and could be successfully passaged and expanded for validation studies (Figure 1). In RT-PCR analyses, primers corresponding to *PRDM1*, *DPPA3*, *IFITM3*, *TERT*, *DDX4* and *beta –actin* were used to confirm the oogonial stem cell profile of the cultured cells. Relevant controls included oocyte preparations, whole ovary tissue, testicular tissue (positive control) and fibroblasts (negative control).

Since the samples collected prior to the awarding of the grant comprised a large cohort of ovaries from younger pre-menopausal women, we postponed the initial studies described in Aim 1 in which we planned to compare the relative tumorigenicity of oogonial stem cells derived from pre and post menopausal women. We therefore initiated the experiments described in Aim 2 (see below) to prevent further delay of the proposed study.

Subtask 1b: The cells will be virally transduced to stably express luciferase, and then injected into the intraperitoneal cavity of immunocompromised (NOD/SCID) mice. The injections will be done in a dilution series, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$ and $1x10^1$, 2 mice per patient per dilution for a total of 100 mice. Cells will also be injected ($1x10^5$) directly into the ovaries of NOD/SCID (2 mice per patient for a total of 20 mice) mice using a 10 μL NanoFil syringe and a beveled 35gauge needle.

We have generated a lentivirus that expresses both GFP and luciferase and have recently infected pools of oogonial stem cells. Once we have obtained a sufficient number of cells in culture, we will use cell sorting to isolate GFP-positive lentivirus infected cells. These cells will be re-plated for expansion in culture and the expanded cells will then be introduced into immunocompromised NOD/SCID mice by intraperitoneal injection as described in Subtask 1b. This strategy will also allow the completion of Subtasks 1c-1e.

Subtask 1c: Tumor development, growth and metastatic potential will be evaluated by the Mouse Imaging Program (MGH/Harvard core facility). The rate of tumorigenesis as well as metastatic spread will be assessed. When tumors have reached a sufficient size for evaluation, mice will be sacrificed and tumors harvested. Each tumor will be divided into 3 equal pieces, one will be preserved in paraformaldehyde and processed for histological analysis, one snap-frozen for genomic analysis, and one vitrified and stored in liquid nitrogen for further analysis at a later date.

This subtask has not been initiated.

Subtask 1d: For histological analysis as well as immunohistochemistry, PFA-fixed tumors will be embedded in paraffin, sectioned, placed onto slides and 5 sections per tumor will be stained with hemotoxylin and eosin for phenotypic analysis. Additionally, sections will be analyzed for p53, cytokeratin (CK) 7, CK20, PAX-8, WT-1, and ER by immunohistochemistry using commercially available antibodies.

This subtask has not been initiated.

Subtask 1e: Genomic Assessment of Tumors. Gene array analysis will be conducted on Affymetrix gene chips by our institutional core facility. The resulting information will be compared to published data sets.

We also proposed to compare the genomic profile of the tumors generated from the oogonial stem cells with the profile of xenografts derived from primary human serous ovarian cancer and/or primary human serous peritoneal cancer. Toward this goal, we have generated xenografts from such primary tumors which have been harvested and frozen for future nucleic acid isolation.

SAMPLE	PRIMARY SITE	HISTOLOGY	GRADE	Ddx4- CD133-	Ddx4+ CD133-	Ddx4- CD133+	Ddx4+ CD133+
OVCA1	Peritoneum	Serous	3	83.27	7.43	8.89	0.41
OVCA2	Peritoneum	Serous	3	73.1	10.6	9.04	7.26
OVCA3	Peritoneum	Serous	3	92.7	6.41	0.77	0.078
OVCA4	Ovary	Serous	3	69.1	26.1	3.69	1.05
OVCA5	Peritoneum	Serous	3	92.7	3.02	4.08	0.14
OVCA6	Peritoneum	Serous	3	86.9	11.9	0.71	0.41

Table 1. Flow cytometric analysis of Ddx4 and CD133 expression in human ovarian cancer xenografts. The relative frequency (indicated as percent of the total viable population) of cells with the indicated Ddx4 and CD133 expression phenotypes was determined in xenografts derived from six independent primary ovarian serous cancers. Note the consistent presence of a Ddx4+CD133- population in all analyzed samples.

In parallel analyses, we used flow cytometry to determine the relative frequency of Ddx4-positive cells in a subset of xenograft tumors derived from primary human ovarian serous cancer. We detected a Ddx44-positive population within every analyzed tumor (Table 1, Figure 2). The presence of this rare sub-population within human ovarian tumors has never been reported and its biological significance remains to be determined. We have begun to test the relative tumorigenicity of tumor-derived Ddx4+ cells. Specifically, we have isolated purified populations of Ddx4+CD133-, Ddx4-CD133+, Ddx4+ CD133+ and Ddx4-CD133- cells and have injected them into NOD/SCID mice in a limiting dilution assay (Table 2). Tumor formation is currently being monitored in the injected animals.

Task 2: Assessment of induced genetic mutations in human OSCs. Human OSCs from women will be expanded in vitro (see Subtask 1a). These cells will be retrovirally transformed to stably express either genetic mutations or constitutively active oncogenes. The genetically mutated cell lines will be injected into immunocompromised mice and evaluated for alterations in tumorigenic characteristics.

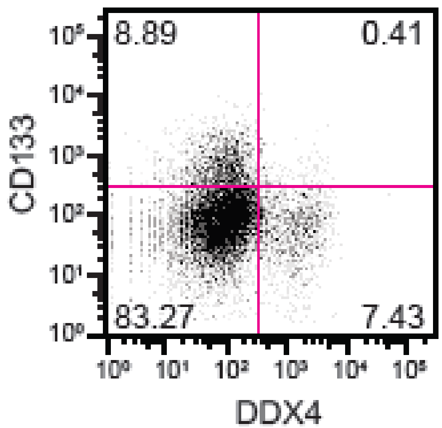

Figure 2. Representative flow cytometry analysis. The distribution of OVCA6 tumor cells across the Ddx4 and CD133 expression profiles listed in Table 1.

	5×10^4	5×10^3	5×10^2
Bulk	4	4	4
Ddx4-CD133-	4	4	4
Ddx4+CD133-	4	4	4
Ddx4-CD133+	2	6	4
Ddx4+CD133+	1	3	8

Table 2. Assessing the relative tumorigenicity of the isolated cell populations. Immunocompromised NOD/SCID mice were injected with the indicated cell numbers of each purified cell population in a limiting dilution assay. The numbers in each column represent the total number of mice injected with each cell number. The information reported here comprises three separate injection series using cells derived from three independent patient xenografts. The bulk population represents unsorted tumor cells.

Subtask 2a: Retroviral transduction of human OSCs. OSCs transformed with luciferase in Subtask 1b will be stably transduced using the pBABE retroviral vectors already produced in our laboratory (dominant negative TP53, KRAS mutant, constitutively active PIK3CA and AKT, and control vector; see preliminary data) using the PLAT-A packaging cell line.

Retroviral vectors expressing oncogenic alleles of human *TP53*, *AKT*, *KRAS*, and *PIK3CA* were constructed and initially validated in both a human endometrial cancer cell line and mouse oogonial stem cells. The expected effect on the expression of relevant genes/proteins was confirmed by RT-PCR or immunoblotting. The viruses were then used to infect a human oogonial stem cell line. Cells infected with the *AKT*, *KRAS* or *PIK3CA* viruses were generated by puromycin selection five days post-infection and then maintained in a low concentration of puromycin to ensure a stable pure population of virus-containing cells. Since the p53-expressing retrovirus construct does not confer puromycin resistance, successful infection with this virus was confirmed by RT-PCR using primers designed to specifically detect exogenous *TP53* gene expression.

Although co-infection of human oogonial stem cells with the oncogenic *TP53* and *KRAS* retroviruses was highly efficient, co-infection of the *TP53* virus with either the oncogenic *AKT* or *PIK3CA* viruses was unsuccessful. We determined that initial infection with the TP53 retrovirus followed by subsequent re-infection with the *KRAS*, *AKT* or *PIK3CA* retrovirus was required to generate human oogonial cells that co-expressed the relevant oncogenes as validated by either RT-PCR or immunoblotting.

Subtask 2b: The transformed cell lines (5 lines) will then injected into the intraperitoneal cavity of

immunocompromised (NOD/SCID) mice. The injections will be done in a dilution series, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$ and $1x10^1$ (3 mice per mutation, for a total of 60 mice).

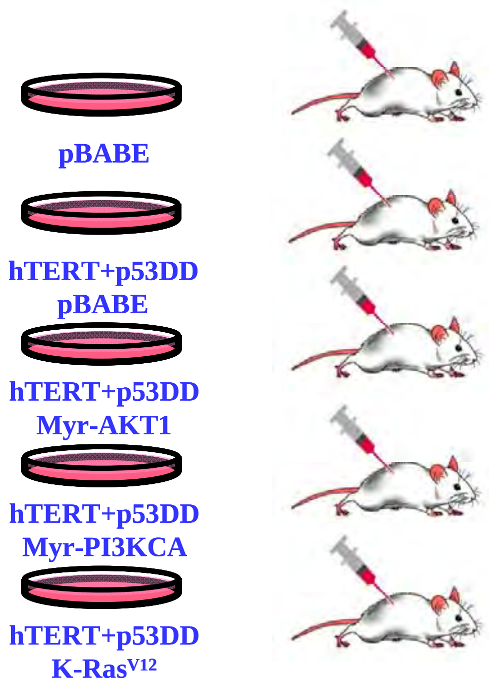

Figure 3. OSC injection into NOD/SCID mice. OSC expressing dominant negative p53 either alone or in combination with activated Akt, PI3K or K-Ras were injected into NOD/SCID mice in a limiting dilution assay. OSC cells infected with parent virus alone (pBABE) were also injected. Tumor formation in the injected mice is currently being monitored.

We conducted simple preliminary studies to assess the potential impact of the retroviral infections on human oogonial stem cell proliferation. Surprisingly, human oogonial stem cells expressing exogenous oncogenic p53, K-Ras, Akt or PI3K reached confluency in culture 3-fold faster than cells infected with the control retrovirus. We have since subcutaneously injected varying cell numbers (1 x 10^6, 1 x 10^5, 1 x 10^4, and 1 x 10^3) of human oogonial stem cells infected with the various retroviruses into NOD/SCID to pre assess their ability to generate tumors in mice. This will inform our choice of the post-injection time points at which we will assess tumor formation in mice using the more expensive IVIS imaging system. Concurrently, we have introduced the co-expressing GFP and luciferase lentivirus into the retrovirus-infected human oogonial stem cell lines. Following lentiviral infection, the cells will be sorted based on GFP expression to generate pure populations of infected cells. Those cells will subsequently be injected into the intraperitoneal cavity of NOD/SCID mice and tracked *in vivo* via IVIS imaging of luciferase activity.

Subtask 2c: The transformed cell lines (5 lines) will then injected into the intraperitoneal cavity of immunocompromised (NOD/SCID) mice. The injections will be done in a dilution series, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$ and $1x10^1$ (3 mice per mutation, for a total of 60 mice). .

This subtask has not been initiated.

Subtask 2d: Tumor development, growth and metastatic potential will be evaluated by the Mouse Imaging Program (MIP, MGH/Harvard core facility) (All mice will assessed weekly in the IVIS imaging system; Each run takes approximately 20-30 minutes). The rate of tumorigenesis as well as metastatic spread will be assessed by the core facility software. When tumors have reached a sufficient size for evaluation, mice will be sacrificed and tumors harvested. Each tumor will be divided into 3 equal pieces, one will be preserved in paraformaldehyde and processed for histological analysis (including immunohistochemistry), one snap-frozen for genomic analysis, and one vitrified and stored in liquid nitrogen for further analysis at a later date.

This subtask has not been initiated

Subtask 2e: As in task 1, tumors will be analyzed for histological analysis as well as immunohistochemistry. PFA-fixed tumors will be embedded in paraffin, sectioned, placed onto slides and 5 sections per tumor will be stained with hemotoxylin and eosin for phenotypic analysis. Additionally, sections will be analyzed for p53 signature, CK7, CK20, PAX-8, WT-1, and ER by immunohistochemistry using commercially available antibodies.

This subtask has not been initiated.

KEY RESEARCH ACCOMPLISHMENTS:

1. The oogonial stem cell preparations/cultures isolated from both pre and post menopausal ovaries collected prior to receiving funding did not survive long term cryopreservation. Consequently, we had to completely restock our resource of ovarian tissue.

2. We acquired human ovarian tissue from nine different patients via the MGH GYN Tissue Repository.

3. A subset of these has been cryopreserved for future isolation of oogonial stem cells.

4. It was ascertained that the nine samples collected represent ovarian tissue from women considered to be pre and post menopausal. As expected, the majority of the samples are post-menopausal ovaries.

5. Three of the newly collected samples were subjected to flow cytometric sorting. Only two of the three sorted samples yielded viable cells.

6. The two viable oogonial stem cell cultures have been successfully passaged and expanded for validation studies.

7. To validate the oogonial stem cell nature of the isolated cultures, we used primers for *PRDM1*, *DPPA3*, *IFITM3*, *TERT*, *DDX4* and *beta –actin* in RT-PCR analyses. Relevant controls included oocyte preparations, whole ovary tissue, testicular tissue (positive control) and fibroblasts (negative control).

8. Retroviral vectors expressing oncogenic alleles of human *TP53*, *AKT*, *KRAS*, or *PIK3CA* were successfully constructed.

9. The viral constructs were initially validated in a human endometrial cancer cell line and mouse oogonial stem cells. The expected effect of each on expression of appropriate genes/proteins was confirmed by RT-PCR or immunoblot analysis.

10. Once validated, the viruses were then used to infect a human oogonial stem cell line. Cells infected with the *AKT*, *KRAS* or *PIK3CA* viruses were generated by puromycin selection five days post-infection and then maintained in a low concentration of puromycin to ensure a stable pure population of virus-containing cells. As described, the

expected effect on the expression of relevant genes/proteins was confirmed by RT-PCR or immunoblotting.

11. Since the p53-expressing retrovirus construct does not confer puromycin resistance, successful infection with this virus was confirmed by RT-PCR using primers designed to specifically detect exogenous *TP53* gene expression.

12. Simple preliminary studies assessing the potential impact of exogenous expression of oncogenic alleles of human *TP53*, *KRAS*, *AKT* and *PIK3CA* have determined that human oogonial stem cells expressing any of these proteins reach confluency in culture 3-fold faster than cells infected with the control retrovirus.

13. In order to compare the genomic profile of tumors generated form human oogonial stem cells to the profile of xenografts derived from human primary serous ovarian or peritoneal tumors, we have generated xenografts from which have been harvested and frozen for future nucleic acid isolation.

14. Using flow cytometry, we have detected a population of Ddx4-positive cells in xenografts derived from primary human serous ovarian and peritoneal tumors. This rare sub-population has never been reported and its biological significance remains unclear.

REPORTABLE OUTCOMES:

- *manuscripts, abstracts, or presentations*

  No manuscripts

- *licenses applied for and/or issued;*

  No licenses have been applied for or issued

- *degrees obtained that are supported by this award;*

  N/A

- *development of cell lines, tissue or serum repositories;*

  As part of the proposal we have collected samples of ovaries and developed short term cell lines from the oogonial stem cells. Due to IRB restrictions these are not available for distribution.

- *informatics such as databases and animal models, etc.;*

  N/A at this point in time

- *funding applied for based on work supported by this award;*

N/A

- *employment or research opportunities applied for and/or received based on experience/training supported by this award*

  N/A

CONCLUSION:

The work completed to date has determined that the human oogonial stem cells are far more unstable then their murine counterparts and require much more oversight. Nevertheless, our preliminary *in vitro* results indicate that our recently initiated *in vivo* analyses should yield some exciting results. Also of importance is the identification of Ddx4-positive cells in xenografts derived from primary human ovarian serous tumors. The biological significance of this apparent rare population is yet to be determined. We have designed and implemented initial studies to test the relative tumorigenicity of Ddx4-positive, CD133-positive and Ddx4 CD133 double positive fractions.

REFERENCES:

N/A

APPENDICES:

N/A

SUPPORTING DATA:

Figures have been included within the text.